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PURIFICATION AND CHARACTERIZATION OF HEAVY METAL INDUCED PROTEASE FROM PSEUDOMONAS FLUORESCENS ATCC 948

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ABSTRACT

The microorganisms that live in association with plant or animal cells rely extensively on the production of extracellular proteases, secondary metabolites and siderophores for their survival. In the gram-negative bacteria, the expression of extracellular products is controlled by the conserved two-component regulatory system consisting sensor kinase GacS and cognate response regulator GacA that also influences the stress tolerance in these bacteria. Oxidative stress is one of the major reasons for the formation of oxidized proteins in aerobic bacteria. During stress, bacteria adapt to the presence of reactive oxygen species (ROS) and oxidation of proteins, by increasing the expression of detoxifying enzymes, protein and DNA repair molecules, which helps in keeping the concentration of these species to sub toxic level by removing the unwanted proteins. This role is attributed to proteases, as they are known to scavenge these oxidized proteins. In response to heavy *metal* stress (lead, copper and cobalt) in *Pseudomonas fluorescens* ATCC 948, protease increases in culture supernatant. The highest protease activity was found with exposure of culture to lead. The lead-induced protease was purified to homogeneity and was identified as 33kDa metalloprotease. The purified protease was maximally active at 20^{0}C and pH 6.0. The nature of the protease was elucidated using different protease inhibitors, which indicated the protease as metalloprotease. The enzyme is characterized and its role in mechanism of adaptation to heavy metal stress has been discussed.

KEYWORDS: ATCC-American Type Culture Collection; *Pseudomonas fluorescens*; ROS-Reactive Oxygen Species; Metalloprotease, Environmental Pollution;

INTRODUCTION

Heavy metal deposition in terrestrial and hydrosphere is resultant in magnified accumulation rendering toxic remnants persevered for a prolonged duration creating health havoc (Ali et al.,2019 Mathivanan and Rajaram 2014).

Stress management in bacterial bioremediation is the cumulative mechanistic modes comprising morphological changes, formation of metallothionein inside the cell, and production of siderophore, extracellular polymeric substances, outside the cells for defense mechanisms of bacteria to resist the metal toxicity and environmental adaptation. Exemplary and additional mechanisms comprise efflux pump system, enzymatic detoxification, biotransformation of metal ions which confirm the innate mode of stress tolerance to effective management of contamination resultant detoxification Bourles et al.,2020,Rizvi et al.,Wu et al., 2010).

The heavy metals naturally occurring in the earth's crust are ubiquitous and predominant anthropogenic activities like mining and smelting operations, manufacturing in the industrial sector, and utility of metals and metal-containing compounds in the domestic and agricultural field are responsible for diminished environemental safety (Tchounwou et al., 2012)

New biocatalysts active in unusual conditions are looked for among extremophilic microorganisms including psychrophiles and psychrotrophs (Adams *et al*.,1995). The microorganisms that live in the association with plant or animal cells rely extensively on the production of extracellular proteases, secondary metabolites and siderophores for their survival (Blumer et al.,1999). In the gram-negative bacteria, the expression of extracellular products and virulence factors are controlled by the conserved two component regulatory system consisting sensor kinase GacS and cognate response regulator GacA. Pseudomonads are widely distributed in diverse niches and are extremely versatile and adaptable. This adaptability is thought to be associated with their diverse and hierarchical siderophore systems.(Cornelis P and Matthijis S.,2002 Cornelis P 2010).

In the bio-control strain CHAO of *Pseudomonas fluorescens*, the response regulator GacA is essential for the synthesis of extracellular protease (AprA) and secondary metabolites including hydrogen cyanide, since it exerts its control on the AprA gene indirectly via a posttranscriptional mechanism (Blumer *et al*, 1999). These global regulatory genes also influence the stress tolerance in several bacteria (Whistler, 2000). The biochemistry of enzymes produced by *Pseudomonas fluorescens*, such as proteases which are heat stable yet active at low-temperature, has been quite well documented (Andersson et al, 1979; Griffith, et al., 1981; Law, 1979; Lawrence,1967). Proteases scavenge oxidized proteins in number of species (Davies et al., 1988) and one of the major reasons for the formation of oxidized proteins is the stress, which most of the aerobic bacteria experience during their growth. Bacterial encounter with heavy metals causes generation of free radicals (Paik et al., 2003) and other activated oxygen species which cause modifications of the amino acids of proteins that result in loss of protein function/enzymatic activity (Grune et al., 1997). During stress, bacteria try to adapt to the presence of reactive oxygen species (ROS) and oxidized proteins, by increasing the expression of detoxification enzymes, protein and DNA repair functions (Mongkolsuk, 2002) (Sharma et al,2006) which helps in keeping the concentration of these species up to sub toxic level in bacterial cell by removing the unwanted proteins (Jenal, 2003).

Thus the survival of bacteria in high concentration of heavy metals, observed in the study, has been attributed to the high activity of single or various proteases which in turn can be the result of the effect of heavy metal stress on the two component regulatory systems GacA/GacS. The survival strategy of these bacteria also involves the stimulation of protease activities as an important aspect in the mechanism of survival. It is precisely for this reason that the present study involves the purification and characterization of protease from the bacterial cell culture exposed to heavy metal stress.

MATERIALS AND METHOD

Pseudomonas fluorescens ATCC 948 culture procured from IARI, Pusa, Heavy Metals (Pb, Cu and Co), centrifuge, agarose, Coomassie brilliant blue stain, BSA, casein, Tris-HCl buffer, TCA, DEAE Sepharose CL6B column, protease inhibitors (Sigma)

Pseudomonas fluorescens ATCC 948 was grown in the presence and absence of Heavy Metals (Pb, Cu and Co) in citrate mineral media at 28^{0}C with constant shaking at 200rpm, till it reached late logarithmic phase. Bacterial cells were centrifuged at 5000rpm and filtrates were collected. Protease activity was checked in all the culture filtrates (Control, Pb, Cu and Co).

Protease Assay in Culture filtrate

Qualitative Agarose plate assay: The proteolytic activity was analyzed using 0.1% casein as a substrate in 10mM Tris-HCl buffer, pH-8.0. Equal amount of supernatant (Control, Pb, Cu and Co) was incubated in the wells perforated on 1% agarose plate and incubated at 37^{0}C overnight. Plate was stained with Coomassie brilliant blue for fifteen minutes followed by destaining.

Quantitative Spectrophotometric assay: The crude protein from control and cultures exposed to, lead, copper and cobalt was also checked for its proteolytic activity quantitatively. A portion of 0.1% casein in 10mM Tris-HCl buffer, pH-8.0 was incubated with 10 l of the extracellular protein extract at 37^{0}C for half an hour. Reaction was stopped by adding 4% TCA. After centrifugation, protein estimation was done using Bradford assay for the soluble peptides in the supernatant of control and treated cultures.

Purification of protein

The crude protein showing highest protease activity in the supernatant (from Pb) was subjected to 90% ammonium sulphate precipitation, 4^{0}C overnight. The precipitate obtained was dissolved in water and dialyzed overnight against double distilled water. The dialyzate was lyophilized and suspended in 50mM

Tris-HCl, pH-8.0 followed by protein estimation with Bradford's Method. The crude protein (1.4 mg) was loaded on the DEAE Sepharose CL6B column, previously equilibrated with the 50mM Tris-HCl, pH-8.0. Washing fractions were collected with the same buffer followed by elution of bound proteins with 200mM NaCl in 50mM Tris-HCl, pH-8.0. Protease activity was checked for both the fractions by agarose plate assay. Fractions containing protease activity were pooled, dialyzed against 10mM Tris-HCl, pH-8.0 and lyophilized.

Characterization

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Lyophilized unbound and bound protein was resolved on 12% SDS-PAGE under reducing conditions by the method of ***Laemmli*** and checked for its purity.

Proteolytic activity of Purified protein

Proteolytic activity of purified protein was analyzed qualitatively by agarose plate assay (as mentioned earlier) and also quantitatively by spectrophotometric assay.

Substrate Specificity: The protease activity of purified protein was analyzed using three different substrates –BSA (1mg/ml), Casein (0.1%) and Defatted milk (1%) from SIGMA. The assay was carried out as mentioned earlier.

Optimum Protein Concentration: Purified protease was incubated in varied amounts, with casein (0.1%) substrate at 37^{0}C for 30 minutes to elucidate the optimum amount of protein needed for protease activity. The proteolytic activity was then measured as described above.

Optimum pH: Purified protein (5µg) was incubated with substrate, casein (0.1%) and dissolved in 10mM acetate-buffer (pH 4 and 6) and 10 mM Tris-HCl buffer (pH 7 and 8). Protease activity was checked using same method.

Optimum Temperature: Purified protein (5µg) was incubated with the substrate at three different temperatures i.e., at 20, 37 and 55^{0}C for half an hour and protease activity was analyzed.

Protease Inhibition Assay: Seven different inhibitors i.e., phosphoramidon, pefebloc, EDTA, PMSF, leupeptin, pepstatin and aprotinin were used in their maximum working concentration to elucidate the nature of purified protease. Purified protease was incubated with inhibitors at 30^{0}C for 20-30 minutes. The assays were performed after adding this reaction mixture with 1 ml of substrate for half an hour at 37^{0}C in the same manner as mentioned above. An appropriate control, without inhibitor was assayed simultaneously.

RESULTS

Protease activity in crude sample (Culture filtrate)

The protein supernatants of heavy metal treated and untreated bacterial cultures showed proteolytic activity in agarose plate assay, which appeared as lightly stained area around the wells. Protease activity was maximum in supernatants of lead treated culture as indicated by its largest zone of inhibition (Fig 1.1). Quantitative assay also confirmed the same results (Fig.1.2)

Purity

The enzyme was purified with an anion exchange chromatography using the DEAE sepharose using the equilibration buffer of pH 8.0. The activity was recovered in washing fractions indicating that around neutral pH the protein is positively charged. The protease activity was checked by agarose plate assay as well as spectrophotometrically. The purity of the purified protease was also analyzed by SDS-PAGE along with molecular weight marker (BIORAD, USA). The single band showed molecular weight of approx. of 33 kDa (Fig. 1.3)

Characterization

Substrate specificity: Among the three substrates analyzed, the purified protein showed the maximum protease activity with casein. (Fig. 1.4)

Optimum Protein Concentration: The purified protein showed optimum activity at the concentration of 5µg. However, the activity was more or less same with 10µg of purified protein. (Fig. 1.5)

Optimum pH: The pH optima required for proteolytic activity was found to be 6.0 using 0.1% casein as a substrate. The activity however decreased at more acidic and basic pH (Fig.1.6)

Optimum Temperature: The purified protein showed maximum proteolytic activity at pH 6.0 with casein as a substrate when incubated at a temperature of 20^{0}C. (Fig. 1.7)

Protease Inhibition Assay: The nature of protease was confirmed by using seven different protease inhibitors in their maximum working concentration. Maximum inhibition was shown with phosphoramidon (89.01 %) followed by leupeptin (85.71%) and pepstatin (80.21%) which indicates that it may be metalloprotease. (Fig.1.8). The result is described as percentage inhibition of proteolytic activity.

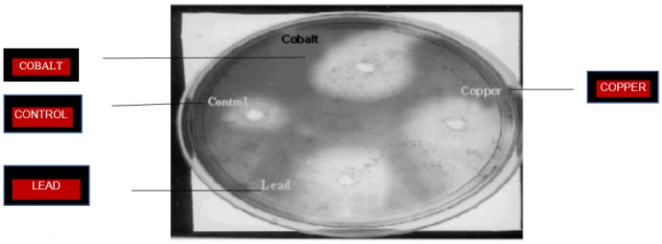

Figure 1

Qualitative analysis using agarose plate assay. Equal amount of protein was added in all the wells using 0.1% casein as a substrate. Well containing supernatant of lead treated culture has shown the maximum zone of inhibition

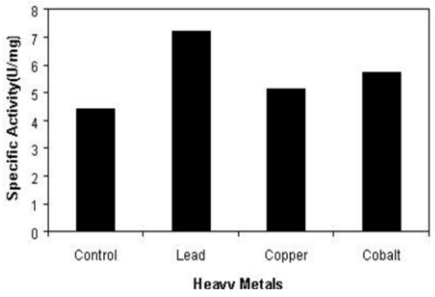

Figure 2

Quantitative analysis. Crude protein from culture supernatants of control as well heavy metal exposed culture. Lead treated culture has shown the maximum activity (7.19 U/mg).

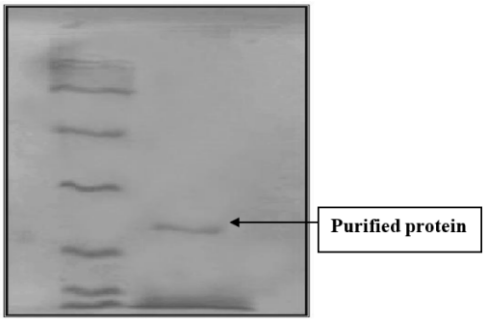

Figure 3

Molecular weight analysis. SDS-PAGE (12% resolving) profile of 33 kDa purified protein along with broad range molecular weight marker (BIORAD). The protein was stained with Coomassie brilliant blue. Lane1-Molecular Weight Marker Lane 2- Purified Protein

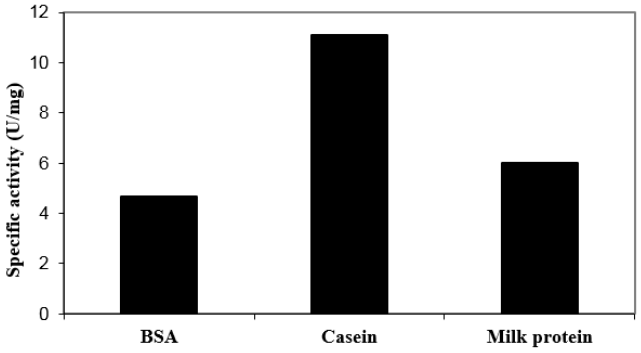

Figure 4

Proteolytic activity of purified protein. Three different substrates i.e., BSA (1mg/ml), casein (0.1%) and defatted milk (1%) were used. The protein (5µg) was incubated with three different substrate solutions for 30 min at 37^{0}C. The cleaved protein substrate was precipitated with 4% TCA and absorbance (Bradford assay) of soluble peptides remaining in the solution was measured with respect to substrate blank.

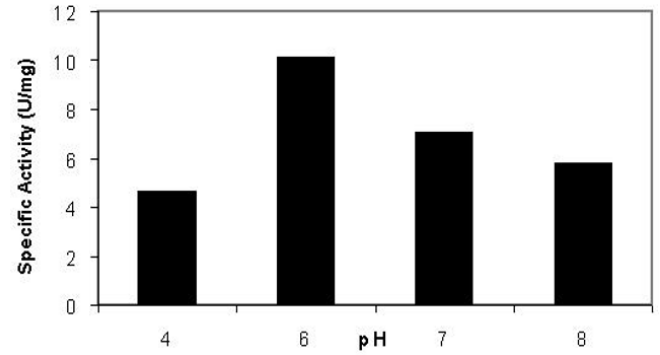

Figure 5
Proteolytic activity using varying amount of protein. The protein (2-15 µg) was incubated with constant amount of substrate (casein) at 37°C for 30 min.

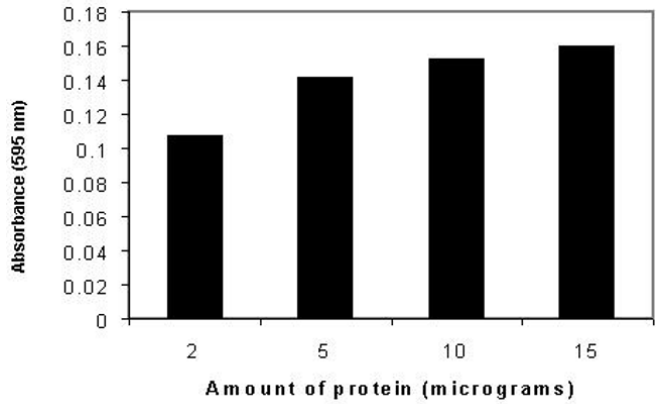

Figure 6
pH optimization. pH optima required for maximum proteolytic activity of *P.f.* ATCC 948 using casein as a substrate. The protein was dissolved in Tris-HCl buffer (0.01 mM) of pH range 4-8 and incubated with the substrate at 37°C for 30 min.

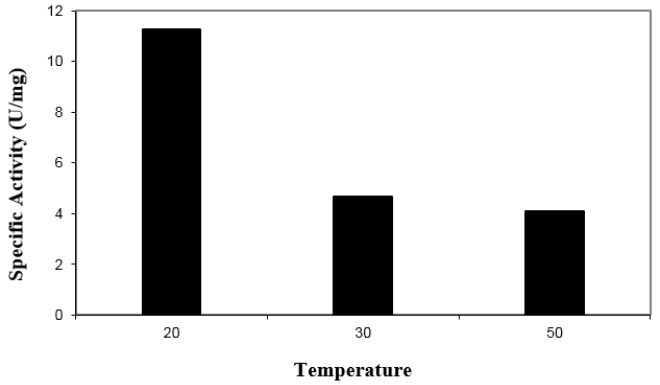

Figure 7
Temperature optimization. Temperature optima of purified protein incubated at three different temperatures i.e., 20°C, 30°C and 50°C for 15 min followed by its proteolytic activity by incubation at 37°C for 30 min.

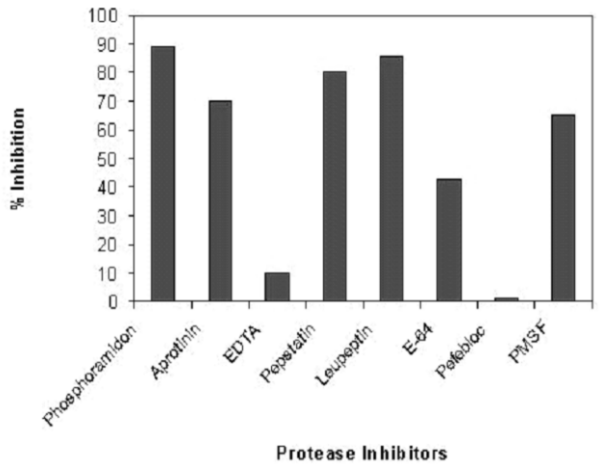

Figure 8
Percentage inhibition. Seven inhibitors were used to detect proteolytic activity. The purified protein (5µg) was preincubated for 20 min at 30°C with maximum working concentrations of different protease inhibitor. This reaction mixture was then incubated with 1 ml of substrate at 37°C for 30 min. The reaction was stopped with 4% TCA and absorbance of cleaved substrate was taken at 595nm with respect to uninhibited control protein.

DISCUSSION

Biochemical characterization of proteases from several bacteria including Pseudomonads is being carried out over two decades now (Alichanidis, 1977; Mitchell, 1989; Stepaniak et al., 1982). Several reports have suggested the role of proteases in the removal of oxidized, nonfunctional proteins (Jenal,2003) which is not reported for this bacterium. These oxidized proteins, more often, are useless owing to their non- functional nature. The proteases probably recognize oxidized proteins by hydrophobic amino acid residues, aromatic residues and bulky aliphatic residues that are exposed during the oxidative rearrangement of secondary and tertiary protein structure. As increased surface hydrophobicity is a feature common to all oxidized proteins so far tested, the recognition of such (normally shielded) hydrophobic residues is the suggested mechanism by which proteases catalyze the selective removal of oxidatively modified cell proteins. By minimizing protein aggregation and cross-linking and by removing potentially toxic protein fragments, proteases play a key role in the overall antioxidant defense mechanism (Grune et al., 1997). We have not come across any report related to the effect of heavy metal exposure on the proteolytic activity till now. However, as an adaptive response to over all stress, high expression of lon, is reported in *Pseudomonas fluorescens* Pf-5 during exponential phase (Whistler et al., 2000) which encodes ATP-dependent serine proteases (Gottesman, 1996; Gottesman et al, 1992). In E. Coli., *lon* is a heat shock protein that nonspecifically degrades denatured non-functional proteins (Gottesman, 1996; Gottesman et al., 1992). In *Bacillus subtilis, lon* expression is induced by salt and oxidative stress as well as by starvation (Riethdorf et al., 1994). Since it is known that Gac A function is required for the expression of protease activity (Sacherer et al., 1994), heavy metal stress acted as an external or internal factor signaling the induced expression of the GacA gene and in turn the increased protease activity. This increased protease activity contributed for the bacterial survival in this heavy metal stress condition by the effective removal of oxidized proteins. To elucidate the nature of protease, we purified and characterized this enzyme. Accordingly, the bacteria were grown as a control and in the presence of lead. The protease activity was found to be higher in bacteria grown in the presence of lead as compared to control. The enzyme was purified from the Lead treated culture. Single step purification has yielded the protease of homogeneous purity, by ammonium sulphate precipitation and DEAE ion exchange chromatography. The purified protease was recovered in the washing fractions as an unbound protein, not necessitating the multistep purification. It may be added here that the process of purification of this protease is our own method and has not been reported anywhere. Till now, various methods involving several steps of purification have been employed to purify proteases from Pseudomonas fluorescens as mentioned earlier but in our study the above-mentioned protease has been purified in a single step showing no affinity with the column used. The purified protease was tested for its activity with the three substrates, of which it showed maximum activity with casein and least with defatted milk, so casein was selected as a substrate for further experiments. The amount of protein used for experiments was 5µg as the increase in the concentration of protein had not shown much effect on its activity, therefore indicating the saturation of substrate. The protease digested casein at pH ranging from 4-8 with maximum at pH 6.0 and temperature at 20°C as compared to 30°C and 50°C. In our studies, the enzyme did not lost its activity even at 50°C, which also indicates the enzyme to be quite thermostable. The proteolytic activity was inhibited mainly by metalloprotease inhibitors in comparison to insignificant inhibition by other type of inhibitors, indicating that the protein we have purified could be a metalloprotease. These observations yield clue towards the important role of protease as a scavenger of oxidized proteins generated during heavy metal exposure. Like in several other organisms, protease performs the role of scavenger of oxidized proteins (Gottesman, 1996; Gottesman et al., 1992), the same holds true for this bacterium also. So, the survival of *Pseudomonas fluorescens* when exposed to heavy metal stress could be attributed to the increased activity of metalloprotease. The relevance of this study has increased due to its applicability in reducing the pollution of water bodies near industries. As reported by Salwan and Sharma 2019., Singh and Bajaj 2017.,Lam et al 2018, proteases are used like detergent additive, contact lens cleaning solution component. These reports further amplify the importance of physiological and commercial roles of proteases. Owing to its single step purification and already identified nature (metalloprotease), further studies at gene level can be done to elucidate the genetic modification due to heavy metal lead. Furthermore, genetic manipulation will also provide a new opportunity to get an insight this bacterial genome using various biotechnological tools to enhance the yield of proteases. (Microbial proteases: ubiquitous enzymes with innumerable uses.(Solankie et al.,2021).

CONCLUSION

The current study is the novel single step purification method for extracting the protease. The protein enhances the ability of Pseudomonas fluorescence to reduce and control the heavy metal contamination in polluted water bodies.

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Authors have no conflict of interest.

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